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Combined chemokine and cytokine gene transfer enhances antitumor immunity

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The probability of producing a specific antitumor response should be increased by multiplying the number of T lymphocytes that encounter the malignant cells. We tested this prediction in a murine model, using a recently discovered T-cell chemokine, lymphotactin (Lptn). This chemokine increased tumor cell infiltration with CD4$^+$ lymphocytes but generated little antitumor activity. Coexpression of the T-cell growth factor interleukin-2, however, greatly expanded the T lymphocytes attracted by Lptn, affording protection from the growth of established tumor in a CD4$^+$ and CD8$^+$ T cell-dependent manner. Lesser synergy was seen with GM-CSF. Hence coexpression of a T-cell chemokine and T-cell growth factor potentiates antitumor responses *in vivo*, suggesting a general strategy to improve cancer immunotherapy.

The introduction of genes encoding diverse types of immunostimulatory molecules into tumor cells can enhance the host's antitumor immunity[1–13]. Because the response generated can be directed against nontransduced (parental) tumor cells, expression of these immunostimulatory molecules can protect against challenge with parental tumor, but only rarely does it induce rejection of preexisting malignant cells[5,10,11]. Most of these efforts to augment immune responsiveness have concentrated on the transfer of genes encoding immunostimulatory cytokines[1–9] or accessory molecules[10–13]. As for any other immune response, however, generation of a specific antitumor response by gene transfer requires that the antigens of interest be presented to T cells that possess the appropriate receptor specificity. Hence, the probability of generating an effective immune response should be increased if one could increase the number and range of host T lymphocytes that encounter the relevant tumor antigen.

Chemokines are molecules that are thought to be capable of attracting effector cells to the sites of inflammatory or immune responses. Lymphotactin (Lptn), a recently discovered addition to the chemokine family[14,15], induces T-cell migration *in vitro* in the absence of any chemotactic effects on either monocytes or granulocytes. We hypothesized that combined delivery of a T-cell chemokine (Lptn) and a T-cell lymphokine (interleukin-2, or IL-2)[3,8,16–19] in the region of an antigen-expressing tumor cell would accelerate and potentiate the antitumor immune response over that elicited by either agent alone. We describe results confirming that prediction in a murine A20 leukemia tumor model.

Effects of lymphotactin on tumor immunogenicity

For this study of the effects of lymphotactin on tumor immunogenicity, we used the murine A20 tumor. This pre-B cell tumor line derives from a spontaneous neoplasm in a BALB/c mouse[20,21]. It is MHC class II positive (and MHC class I inducible), but has little or no inherent immunogenicity in syngeneic recipients[20,21], so that repeated immunization of BALB/c mice with irradiated tumor cells provides no discernible protection against subsequent challenge with live malignant cells (Table 1).

We first asked whether paracrine secretion of Lptn would increase the infiltration of cytotoxic T lymphocytes into developing tumors. Tumor specimens from mice that had been injected subcutaneously with both A20 cells and fibroblasts secreting Lptn showed distinct and uniformly distributed tumor infiltrates of CD4$^+$ T lymphocytes (Fig. 1*c*), in contrast to those from mice given *neo*^{R}-transduced (control) fibroblasts, in which only occasional infiltration of CD4$^+$ cells was seen (Fig. 1*a*). CD8$^+$ lymphocytes were present in scanty numbers in control tumors (Fig. 1*b*) and infiltration was not increased in the presence of Lptn-secreting fibroblasts (Fig. 1*d*).

Despite the ability of Lptn to recruit T lymphocytes into growing tumors, results with two models of tumor inhibition indicated that the chemokine had only limited efficacy as an antitumor agent, unless it was combined with IL-2. In the first model, 10^5 parental-type A20 tumor cells were injected with syngeneic fibroblasts (2×10^5) that had been transduced with a control (*neo*^{R} alone) vector or with vectors encoding neo^R and either IL-2 or Lptn. Tumor volumes were determined from three-dimensional caliper measurements, and mice were killed when the tumor mass exceeded 30% of body weight or when there was evidence of ulceration or distress. The rate of tumor development differed among mice depending upon the combination of tumor cells and IL-2- or Lptn-secreting fibroblasts (Fig. 2). Lptn-transfected fibroblasts lacked an antitumor effect, whereas the IL-2-transduced fibroblasts were associated with limited retardation of tumor development ($P < 0.001$ for comparison with the neo^R control group). The most striking inhibitory effect was produced in animals inoculated with both

Table 1 Immunization with A20 tumor does not protect against tumor challenge

	Tumor size (mm^3) for days of study	
	32 days	39 days
Immunized	574 ± 347	2177 ± 1262
Nonimmunized	195 ± 117	1974 ± 871

Mice were immunized with 10^5 irradiated A20 cells on days 0 and 7 and inoculated with 10^5 wild-type A20 cells on day 14. The control group received the tumor challenge alone (see the Methods section). Data show tumor size (±s.d.).

Lptn- and IL-2-secreting fibroblasts ($P < 0.0004$ for comparison with the IL-2 only group)..

Effects of lymphotactin and IL-2 on preexisting tumors

We then tested the ability of Lptn to potentiate IL-2 activity in mice with preexisting tumors, a setting more closely related to the clinic. Mice were injected with 10^5 parental tumor cells. Four days later, they received a mixture of 10^5 parental-type A20 tumor cells and 2×10^5 transduced fibroblasts irradiated before injection (1000 cGy). Immunization was repeated after another 10 days. The results reported in Fig. 3 confirm that IL-2 alone does produce some protection against tumor development ($P < 0.0003$ for comparison with the neo^R control group), and that the addition of Lptn-secreting cells substantially delays the growth of preexisting tumors ($P < 0.02$ for comparison with the IL-2 only group). Consequently, survival is significantly prolonged in the IL-2 group ($P < 0.002$ for comparison with the neo^R group) and still further increased in the IL-2/Lptn group ($P < 0.01$ for comparison with the IL-2 only group) (Fig. 4).

To determine whether the combined effects of Lptn and IL-2 on tumor growth in mice with preexisting leukemia were immunologically mediated, we first analyzed the pattern of tumor infiltration by lymphoid cells in mice receiving the chemokine/cytokine combination. As described (Fig. 1, *a–d*), Lptn by itself induced infiltration only of $CD4^+$ cells. IL-2 alone induced limited infiltration of both CD4- and CD8-positive cells (Fig. 1, *e* and *f*). In contrast, the combination of IL-2 and Lptn lead to extensive and uniform infiltration with both $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 1, *g* and *h*). To confirm the importance of T cells in the antitumor activity of Lptn and IL-2, we injected animals with wild-type tumor, followed on days 4 and 14 by irradiated tumor cells and syngeneic fibroblasts transduced as in earlier experiments. Animals were treated with depleting doses of either CD4 or CD8 antibodies. Depletion of either $CD4^+$ or $CD8^+$ cells abrogated the protective effects of the Lptn/IL-2 combination on tumor growth (Fig. 5) and murine survival (Fig. 4). Hence, an intact CD4 and CD8 system is required for successful immunotherapy in this tumor model.

Lymphotactin and interleukin-2 compared with GM-CSF

Finally, we compared the antitumor effect mediated by Lptn/IL-2 with the activity of granulocyte-macrophage colony-stimulating factor (GM-CSF). Unlike Lptn and IL-2, GM-CSF does not act directly on T lymphocytes but is nonetheless highly effective in many tumor immunization studies[5,22]. In the tumor progression model, production of GM-CSF had limited effects on tumor cell growth that did not reach statistical significance (Fig. 6) and were inferior to the combination of IL-2 and Lptn ($P < 0.0005$ for Lptn + IL-2 vs. GM-CSF). Tumor growth in the mice receiving the GM-CSF immunogen could be further retarded by addition of Lptn (Fig. 6), but the level of protection remained below that obtained when Lptn was combined with IL-2 ($P < 0.005$ for IL-2 + Lptn vs. GM-CSF + Lptn). The prolongation of survival by these agents followed the same pattern (Table 2).

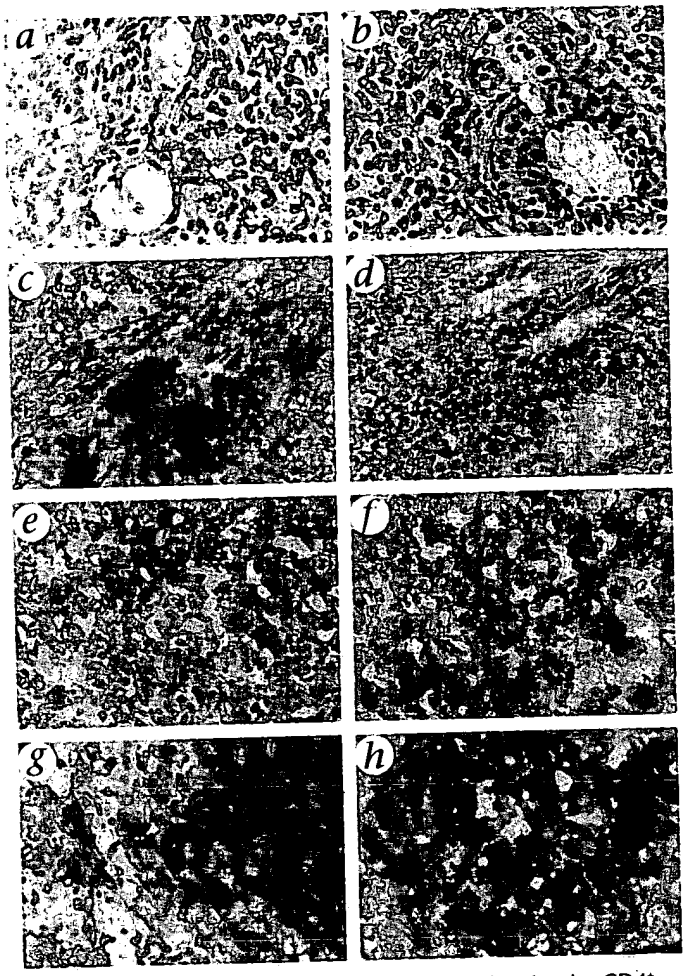

Fig. 1 Effects of Lptn and IL-2 on tumor infiltration by $CD4^+$ or $CD8^+$ T lymphocytes. Mice were injected s.c. with a mixture of 10^5 A20 and 2×10^5 transduced CL7.1 fibroblasts expressing either the neo^R gene (control, *a* and *b*), the Lptn gene (*c* and *d*), the IL-2 gene (*e* and *f*) or both the Lptn and IL-2 genes (*g* and *h*). Tumors were excised when they attained a volume of 3000 mm^3. *a* and *b*, Minimal infiltration of control tumors by either $CD4^+$ or $CD8^+$ lymphocytes. *c* and *d*, Infiltration of the Lptn-secreting tumor with $CD4^+$ cells (*c*) in the absence of an increase of a $CD8^+$ cell infiltrate (*d*). With IL-2, there is a marginal increase in both $CD4^+$ (*e*) and $CD8^+$ (*f*) T lymphocytes compared with controls, but in the IL-2 and Lptn group, there is a massive infiltrate of both $CD4^+$ and $CD8^+$ T lymphocytes (*g* and *h*).

Discussion

Chemokines may play a critical role in recruiting effector cells for inflammatory and immune responses[23,24]. *Ex vivo* experiments have suggested that the chemokine Lptn is particularly important for the recruitment of T cells to the site of an immune response[14,15]. We have provided the first *in vivo* evidence that

Table 2 Prolongati n of survival in GM-CSF/Lptn-treated mice

Additional days survival vs. controls		
GM-CSF	GM-CSF + Lptn	IL-2 + Lptn
2.4 ± 1.8*	7.3 ± 3.1**	22.9 ± 5.4***

Values are means ± s.d. *No significant prolongation compared with control. **$P < 0.05$ for comparison between GM-CSF and GM-CSF/Lptn groups. ***$P < 0.003$ for comparison between GM-CSF/Lptn and IL-2/Lptn groups.

such T-cell recruitment can indeed be achieved using a chemokine. Murine Lptn has sequence similarities to the Cys-Cys class of chemokines, but maps to a different chromosome. It may therefore represent an entirely separate molecular class[14]. One likely function of Lptn is to widen the T-cell repertoire at the site of an immune response[14,15]. This may be crucial in antitumor responses, where the frequency of cytotoxic precursor cells recognizing the limited range of tumor-associated epitopes may be only 1 in 10,000 to 100,000. We have attempted to exploit this function in a therapeutic strategy by combining the delivery of Lptn with IL-2 (ref. 3, 8, 19). We chose IL-2 in this combination, because the ability of the cytokine to expand T-cell clones following engagement of their antigen-specific receptor appeared to complement the known cellular selectivity of lymphotactin[3,8,16–18,25]. Moreover, administration of IL-2 has produced responses in human leukemias[26,27], increasing the relevance of this model to the human disorder.

The efficacy of this attraction-expansion approach was striking by comparison with the less pronounced antitumor protection provided by either agent alone. Our experience showing the limited protective effects of using IL-2 alone are consistent with most previous results using single agents[1–13]. The failure of many single agents to suppress preestablished malignancy may result either from a delay in generating a response or from a failure to recruit cells of the appropriate specificity. The combination of the T-cell chemokine (Lptn) and a lymphokine (IL-2) has the potential to overcome both limitations.

By itself, Lptn produces an increased $CD4^+$ infiltration of the tumor cells. Although scanty CD8 cells were present, their numbers were not increased compared with those of controls. This observation was at variance with our expectations, as the *ex vivo* activity of the chemokine is predominantly on $CD8^+$ T cells[14,15]. This apparent distinction between *ex vivo* and *in vivo* effects has been confirmed in an independent model in which Lptn is injected into the intraperitoneal cavity of normal mice and induces a T-cell infiltrate in which $CD4^+$ helper T cells are also present (A.Z., unpublished data). The observed increase in T-cell infiltrate alone does not retard tumor cell growth, nor does it prolong the animals' survival. Fibroblast delivery of IL-2, on the other hand, marginally increases infiltration with $CD4^+$ and $CD8^+$ cells, but is insufficient to generate a substantial response against preexisting tumor. The Lptn/IL-2 combination, however, induces marked infiltration of both $CD4^+$ and $CD8^+$ cells and produces significantly greater suppression of preexisting tumor than either molecule alone. Evidently, the immune responses recruited by the chemokine and cytokine individually are insufficiently potent to control the growth of the A20 leukemia cells studied here. We propose that the ability of Lptn to enhance the action of IL-2 comes, at least in part, from its ability to attract increased numbers of $CD4^+$ T cells to the tumor site. Although $CD4^+$ cells alone have limited direct antitumor activity, the concomitant presence of IL-2 will allow expansion of any

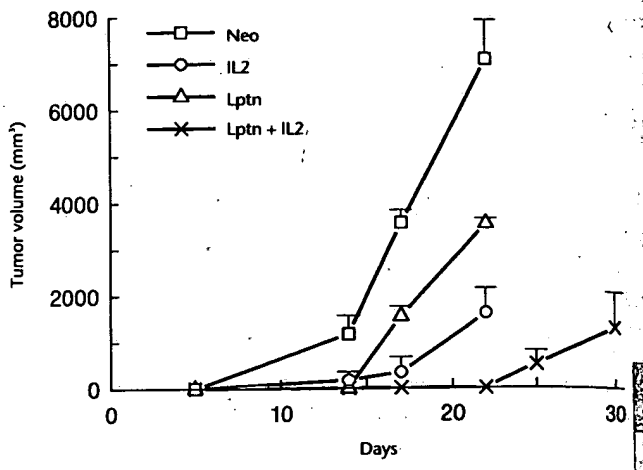

Fig. 2 Effect of gene-modified fibroblasts secreting Lptn or IL-2 o A20 murine leukemia cell growth. Three groups of BALB/c mic (seven per group) were injected s.c. with a mixture of 10^5 A20 cel and 2×10^5 transduced CL7.1 fibroblasts expressing either the Lpt or the IL-2 gene or *neo*[R] (control). In a fourth group of mice, A2 cells were mixed with a combination of fibroblasts modified sepa rately to express Lptn or IL-2. Tumor size is measured by calipers an is reported as cubic millimeters (means ± s.e.m.).

tumor-specific $CD4^+$ T cells, which in turn may be able to am plify the antitumor response mediated by IL-2-dependent CD8 cytotoxic T cells[8]. In the absence of Lptn, IL-2 will have a greatl reduced probability of expanding tumor-specific T-cell popula tions[25] and a correspondingly reduced effect on tumor growth This suggestion is supported by our observation that depletion o either $CD4^+$ or $CD8^+$ T cells depresses the antitumor respons elicited by a combination of Lptn and IL-2.

In the model chosen for study, the combination of Lptn an

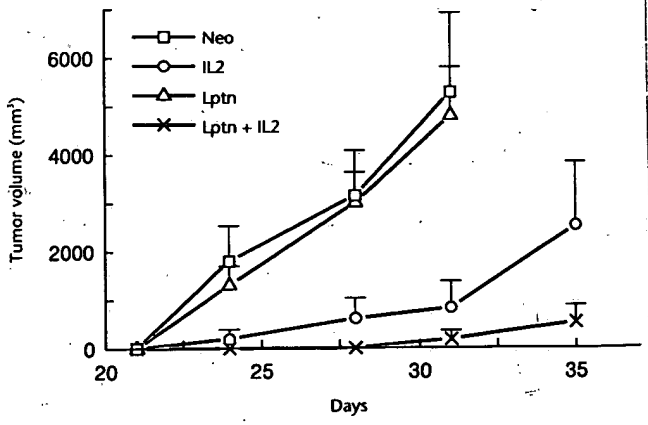

Fig. 3 Effect of irradiated A20 cells mixed with gene-modified fibroblasts expressing Lptn or IL-2 on the growth of preexisting tumor. Four groups of mice (seven per group) were challenged with 10^5 A20 cells on day 1. On days 4 and 14 mice received s.c. injection of 10^5 A20 cells mixed with 2×10^5 transduced CL7.1 fibroblasts expressing either the Lptn or IL-2 gene, or *neo*[R] (control). Before injection, the cell mixture was irradiated to 1000 cGy. The fourth group of mice was treated with irradiated A20 cells and a combination of fibroblasts expressing either Lptn or IL-2. The data are reported as cubic millimeters (means ± s.e.m.).

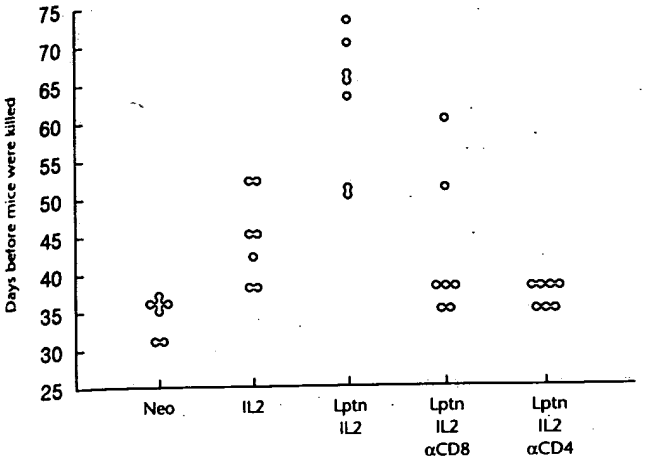

Fig. 4 Injection of irradiated A20 cells mixed with a combination of fibroblasts expressing either Lptn or IL-2 prolongs survival in mice with preexisting leukemia. Each data point represents the survival time of an individual mouse in each group.

IL-2 appears to offer levels of protection that are not only higher than either molecule alone, but are also greater than the protection provided by the single cytokine GM-CSF. In many tumor models, production of GM-CSF alone elicits a significant antitumor response[5,22,28]. Unlike IL-2, GM-CSF lacks primary activity on T lymphocytes. Instead, it improves tumor antigen processing and presentation by its effects on monocytes, macrophages and other professional antigen-presenting cells (APCs). These effects result in turn in enhanced CD4 and CD8 effector function[5,22]. We found GM-CSF afforded no significant protection against the outgrowth of preexisting A20 tumor. Although it is possible that higher levels of GM-CSF secretion would have afforded a higher level of protection[5,16,22], we believe the more significant observation is that the addition of Lptn to GM-CSF enhanced the protection afforded by GM-CSF alone. We do not know what mechanism underlies this effect, but it may result from the exposure of the increased numbers of T cells, recruited by Lptn, to tumor antigens on the professional APCs, recruited by GM-CSF (ref. 5). Whatever mechanism is responsible, the data indicate that the synergistic immunomodulatory activities of Lptn are not limited to cytokines acting directly on T lymphocytes.

In summary, these results demonstrate that local synthesis of the chemokine lymphotactin increases T-lymphocyte migration *in vivo*. Migration alone is not associated with an increase in immunologic activity as measured by an antitumor effect. However, the combination of this T-cell chemokine together with the T-cell lymphokine IL-2 produces a synergistic antitumor immune response. This combination may be therapeutically useful.

Methods

A20 cell lines. The A20 cell line was obtained from the American Type Culture Collection (Rockville, Maryland). This line consists of pre-B lymphoblastoid cells, originally derived from a BALB/c mouse with a spontaneously growing reticulum tumor[20,21]. The tumor was grown in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, Maryland), 10% FCS (BioWhittaker), 100 IU/ml penicillin and 100 µg/ml of streptomycin, 2 mM L-glutamine (BioWhittaker); the CL7.1 fibroblast cell line[29] was maintained in RPMI 1640 (BioWhittaker) with the same supplements.

Vector construction. To generate a retroviral vector containing the murine lymphotactin (Lptn) gene, we used HaTKN as the retroviral backbone[30]. The full-length cDNA pJFEMoLptn of the murine lymphotactin was provided by the DNAX Research Institute[14]. A vector incorporating both the Lptn and *neo*^{R} genes, designated HaLTNTKNeo, was constructed by subcloning the *Xba*I/*Ssp*I fragment of pJFEMoLTN into the *Xba*I/*Eco*RV sites of the commercially

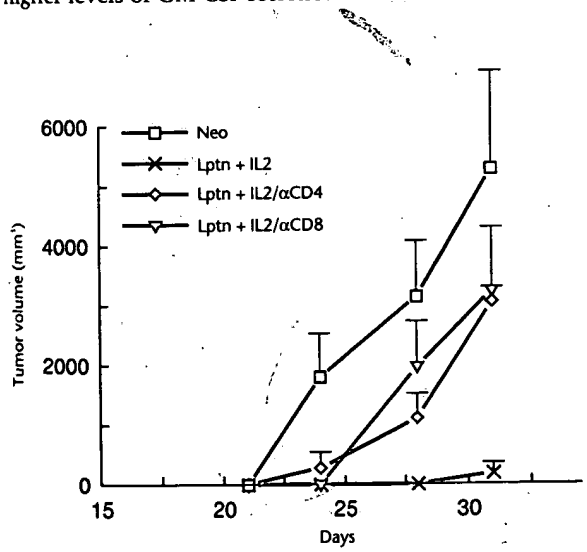

Fig. 5 T cells mediate the inhibition of preexisting leukemia following Lptn/IL-2 costimulation. Four groups of mice (seven per group) were challenged with 10^5 A20 cells on day 1. On days 4 and 14, the animals were treated with irradiated A20 cells mixed with fibroblasts expressing either the Lptn or IL-2 gene or *neo*^{R} (control). Mice were depleted of T lymphocytes by i.p. injection of the Gk1.5 (anti-CD4) or 2.43 (anti-CD8) monoclonal antibodies 1 day before tumor challenge and again on alternate days for 1 week, and then weekly for 4 weeks.

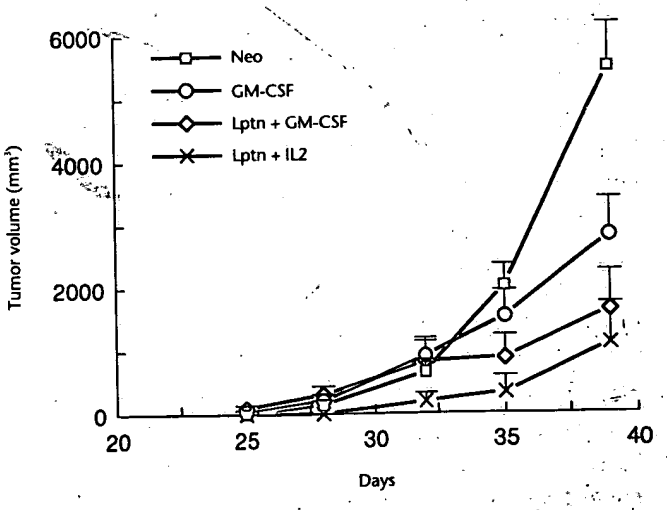

Fig. 6 Granulocyte-macrophage-CSF and Lptn retard preexisting tumor growth. Four groups of mice were challenged with 10^5 A20 cells on day 1. On days 4 and 14, two groups of mice received s.c. injection of 10^5 A20 cells mixed with 2×10^5 fibroblasts (CL7.1) expressing either the *neo*^{R} or GM-CSF genes. A third group received A20 cells and a combination of fibroblasts expressing Lptn and GM-CSF, whereas the fourth group received a combination expressing Lptn and IL-2.

available PSL1180 vector (Pharmacia, Piscataway, New Jersey), which contains multiple cloning sites. The resulting plasmid, PSmLTN, was cut with *NotI*/*SalI*, and the fragment was ligated to the *NotI*/*XhoI* fragment of the HaADHTKN retroviral vector. The producer cell line, BOSC (ref. 31), kindly provided by M. Scott, was transfected with the HaLTNTKNeo plasmid by calcium precipitation, as previously described. Supernatants were collected from the BOSC cells at confluence and then used to transfect the CL7.1 fibroblast cell line. After three rounds of transfection in the presence of 6 μg/ml polybrene, fibroblasts were cultured in selection medium containing the G418 neomycin analogue. Transduction was performed at 37 °C, 5% CO_2 in the presence of 6 μg/ml polybrene. The transduced CL7.1 cells were selected in G418 sulfate (Gibco BRL, Gaithersburg, Maryland) at an active concentration of 1 μg/ml. The neomycin phosphotransferase (*neo*^{R}), human IL-2 (ref. 3, 32) and murine GM-CSF (ref. 5, 22) genes were transferred as previously described[33,34], by using the G1Na, G1NaCvI2 and DISC-GM-CSF vectors (generously provided by Genetic Therapy, Inc., Gaithersburg, Maryland, and Cantab Pharmaceuticals, Cambridge, UK).

Cytokine and chemokine assays. IL-2 production by transduced CL7.1 fibroblasts was 6200 pg/10^6 cells over 24 h, as measured by ELISA (R&D Systems, Minneapolis, Minnesota). Lptn expression, confirmed by RT-PCR, resulted in chemokine concentrations from 1,000 to 10,000 pmol in supernatants from 10^6 transduced CL7.1 fibroblasts, as determined by bioassay[35]. GM-CSF production was 28,000 pg/10^6 cells per 24 h as measured by ELISA.

Tumor growth *in vivo*. Experiments to determine the effects of IL-2, Lptn and GM-CSF on tumor growth were performed in female BALB/cByJ mice aged 12–18 weeks (Jackson Laboratory, Bar Harbor, Maine). This system was syngeneic, as both the A20 and CL7.1 cell lines were derived from BALB/c mice. Cells were washed twice in PBS before injection. Trypan blue-negative cells were adjusted to the desired concentrations in a total volume of 200 μl. Seven mice were included in each treatment group.

For assessment of inhibitory effects on tumor development, 2×10^5 fibroblasts expressing the IL-2, Lptn or *neo*^{R} gene (control) were mixed with 1×10^5 A20 tumor cells and injected subcutaneously (s.c.). For tests against preexisting leukemia, mice were inoculated (s.c.) with 1×10^5 A20 cells on day 1. This was followed on days 4 and 14 by two s.c. injections consisting of a mixture of 2×10^5 fibroblasts, each expressing the IL-2, Lptn, *neo*^{R} or GM-CSF genes, and 1×10^5 A20 tumor cells. Immediately before injection, the cell mixture was irradiated to 1000 cGy. To analyze the inherent immunogenicity of the A20 tumor cells, we challenged two groups of mice with 10^5 tumor cells. One group had received 10^5 irradiated A20 cells on day 0 and 7 before challenge on day 14, whereas the second group was not immunized before inoculation with live tumor.

Tumor volumes were determined by measuring the largest diameter and the respective perpendicular diameter. Tumors with a measurement greater than 10 mm were considered positive. Animals were killed when tumor growth resulted in an increase of more than 30% body weight or in ulceration or distress. Tumor volumes are reported as mean cubic millimeters ± s.e.m. The Wilcoxon test was used to compare the tumor inhibitory effects produced by IL-2 or GM-CSF in the presence and absence of Lptn.

T-cell depletion. Mice were depleted of T lymphocytes by intraperitoneal (i.p.) injection of the Gk1.5 (anti-CD4) and 2.43 (anti-CD8) monoclonal antibodies[36,37] kindly provided by P.C. Doherty. The antibodies were injected i.p. 1 day before tumor challenge and then every other day for a week, followed by weekly injections for 4 weeks. After tumor-bearing animals were killed, the depletion status was 98% as demonstrated by fluorescent-activated cell sorting (FACS) analysis of splenocytes.

Immunohistochemical evaluation. To assess tumor infiltration by CD4$^+$ and CD8$^+$ T lymphocytes, tumors were excised when they reached a volume of 3000 mm^3. Frozen tumor sections were then stained by the alkaline phosphatase-anti alkaline phosphatase (APAAP) technique[38]. Primary CD4 (L3T4) and CD8 (Ly-2) monoclonal antibodies were purchased from PharMingen (San Diego, California). The secondary rabbit anti-rat antibody was obtained from Vector Laboratories, Inc. (Burlingame, California), and the APAAP complex from DAKO (Carpinteria, California).

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